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The critically endangered species *Litoria spenceri* demonstrates subpopulation karyotype diversity

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Abstract.—Litoria spenceri **is a critically endangered frog species found in several population clusters within Victoria and New South Wales, Australia. Biobanking of cell cultures obtained from toe clippings of adults originating from Southern, Northern and Central Site locations, as well as Northern x Central Site hybrid tadpole crosses was performed. Analysis of biobanked cells demonstrates a 2n = 26 karyotype and chromosomal morphology characteristic of the *Litoria* genus. A potential nucleolar organiser region (NOR) on chromosome 9 demonstrates similar designation to *L. pearsoniana* and *L. phyllochroa* of the same phylogenetic subgroup. A second potential novel NOR was also located on the long arm of chromosome 11, and only within the Central Site population. This Central Site apparent NOR is inheritable to Northern x Central Site tadpole hybrids in the heterozygous state and appears to be associated with a metacentric to submetacentric morphological transformation of the Northern Site inherited matched chromosome of that pair. This potential NOR represents an important genetic marker for distinguishing subpopulations. These data demonstrate the importance of prospectively establishing biobanks containing genetically characterized cells so that effective markers of specific subpopulations can be identified and used to help increase the effectiveness of animal husbandry programs.**

Keywords. Frog, biobanking, cryopreservation, cell culture, toe clips, subpopulations, conservation management

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Introduction

The Spotted Tree Frog, *Litoria spenceri* (Spencer 1901), is an IUCN Red List critically endangered amphibian endemic to in Victoria and New South Wales, Australia (Gillespie and Hollis 1996; Skerratt et al. 2016; Fig. 1). *Litoria spenceri* is an obligate stream breeder and historically known to occur at sites between 300–1,100 m elevation (Gillespie and Hollis 1996). The species has been intensively studied, particularly since the early-mid 1990's to: (i) evaluate possible changes in site occupancy and population dynamics across the species range, and (ii) identify factors linked to subpopulation declines. Population decline studies indicate that *L. spenceri* has disappeared from 50% of known historic sites, and is now rare at all other sites (West 2015). Historically, mining activities and other habitat disturbances have influenced the viability of some subpopulations (Gillespie and Hollis 1996; Watson et al. 1991). Today, ongoing population declines are documented to be driven by non-native fish predation of tadpoles and infection by the fungus *Batrachochytrium dendrobatidis*, that is impacting the survival of terrestrial life stages (Gillespie et al. 2015; West 2015). Due to such factors, *L. spenceri* populations are currently restricted to around 25 sites across nine streams (West 2015).

Conservationists at the Amphibian Research Centre in Australia have captured wild *L. spenceri* frogs from several sites to establish captive insurance breeding colonies (e.g., Brannelly et al. 2017). Safeguarding populations from all sites would be ideal because limited genetic analyses suggest that *L. spenceri* populations may form several distinct genetic clusters or evolutionary significant units (Gillespie and Robertson 1998). Distinct genetic traits may relate to a noted regional but not well described phenotypic diversity within this species and,

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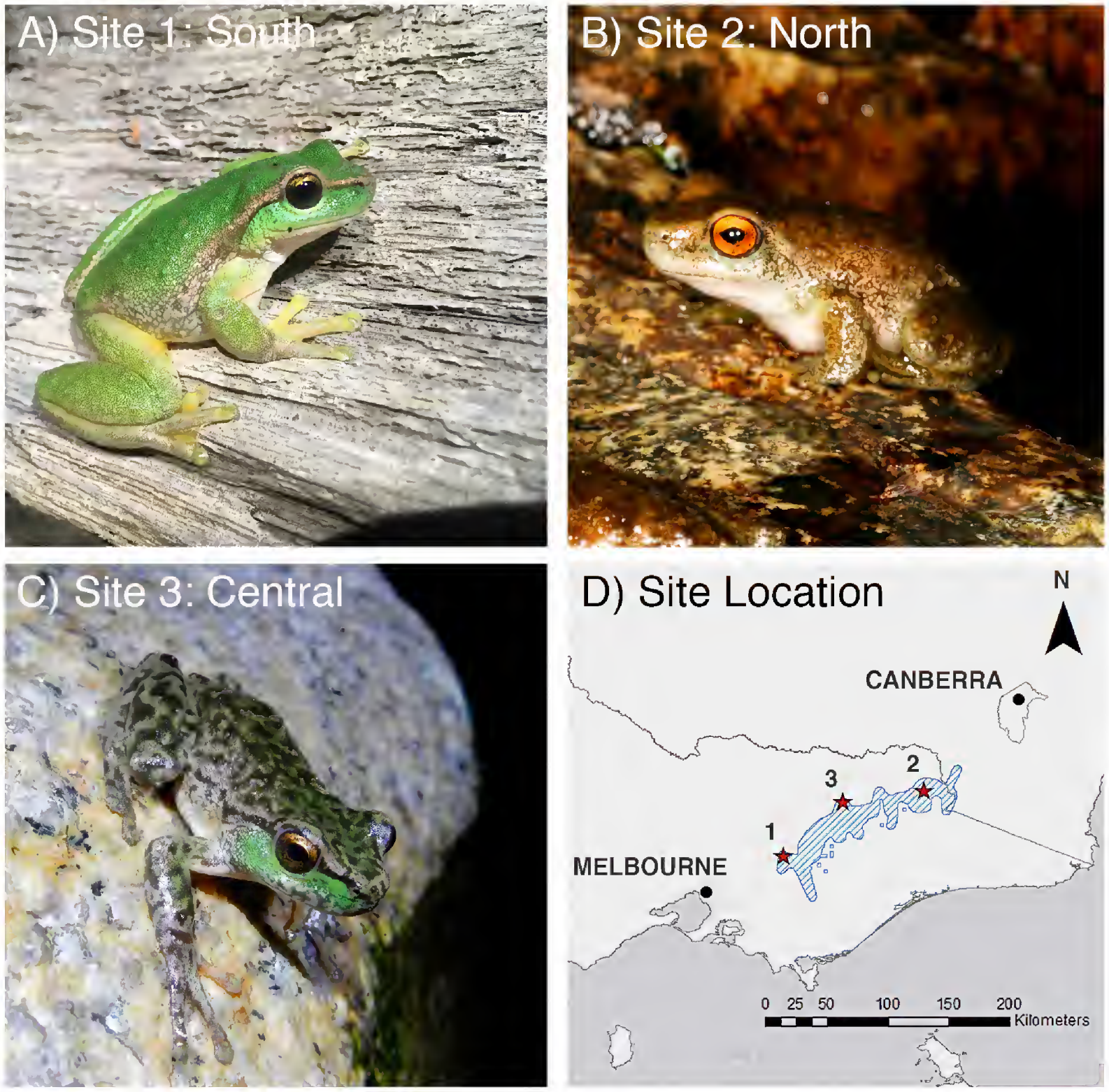

Fig. 1. Phenotypes of *L. spenceri* frogs and site location. **(A)** Adult frog from the South Site (1). **(B)** Adult frog from the North Site (2). **(C)** A juvenile frog from the Central Site (3). **(D)** Site identification within the *L. spenceri* population range. N = north. Phenotypes are only examples and not necessarily representative.

therefore, perhaps contribute to regional survival advantages. Establishing a biobank containing viable somatic cells of all diverse subpopulations would permit genetic mapping of these traits, provide markers facilitating conservation of distinct geographic subpopulations and potentially provide material for assisted reproductive technologies if specific populations were lost (ART; see Clulow and Clulow 2016; Kouba et al. 2013; Mollard 2018 a,b; Zimkus et al. 2018).

Small distal toe tissue samples collected from several *L. spenceri* representative of three diverse population sites within the known population range: Southern, Central and Northern Sites were processed for cell culture and deposited under liquid nitrogen (see West 2015; Mollard 2018a, b). To demonstrate suitability of these cells for future research, samples were thawed and karyotyped. As *L. spenceri* karyotypes were previously not described, inter-site as well as male and female intra-site population comparisons were made. These data suggest strong conservation of karyotypic morphology and chromosome 9 nucleolar organiser region (NOR) between the closely related species *L. pearsoniana* and *L. phyllochroa* (see King et al. 1980). However, DAPI (4',6-diamidino-2-phenylindole) staining demonstrates what appears to be a novel chromosome 11 NOR present only in the Central Site animals. Significantly, when captive Northern Site and Central Site animals were interbred, the novel chromosome 11 NOR is inherited by tadpole offspring in the heterozygous state. Further, the matched chromosome from the Northern Site parent appears to have undergone a metacentric to submetacentric conversion. The study described here provides an example of the essential nature of establishing biobanks of cells, prospectively validated at least at the level of karyotype, for effective species subpopulation management and safeguarding associated diversity.

Methods

Study Design

All tissue from adult and juvenile frogs used within this study was obtained from cultures of toe clippings deposited in the Amphicell Biobank (for previous examples,

see Mollard 2018a, b). Tadpole tissue was obtained from cultures of macerates deposited in the Amphicell Biobank. Clippings from Southern Site female and male frogs were collected on-location at a designated mark-recapture transect (Fig. 1). A toe clipping from a Northern Site non-sexed adult was collected from an adult animal taken from the wild and maintained in captivity for over 15 years (for reference to housing, see Brannelly et al. 2017). Juvenile Central Site derivative animals were bred in captivity as above. The one tadpole was bred in captivity from a Northern Site x Central Site mixed mating as above. All tissues were collected in compliance with relevant State governmental and ethical licensing requirements (for a summary, please see https://frogs.org.au/arc/legal.html), The Code of Ethics of the World Medical Association of The Declaration of Helsinki and the EU Directive 2010/63/EU for animal experiments.

Karyotyping

Karyotyping was performed according to previously described methods (Mollard 2018a,b). Briefly, cryotubes were removed from liquid nitrogen and quickly thawed by rubbing between thumb and forefinger. Cryopreservation media was diluted 10 fold in diluted and supplemented DMEM (Dulbecco's Modified Eagle's Medium; GIBCO) at room temperature up to a total volume of one ml in single wells of a 24 well plate as previously described (Falcon Multiwell™, GIBCO; Ferris et al. 2010; Mollard 2018a). Cells were passaged at approximately 70% confluence into two wells. Cells in both wells were once again grown until approximately 70% confluence. Cells from one well were returned to liquid nitrogen and cells from the other well were processed for karyotyping. Cells for karyotyping were treated with 0.1 µg/ml KaryoMAX® colcemid (GIBCO) for approximately eight hours. Cells thus arrested in metaphase were lifted with 0.25% trypsin/0.02% EDTA, centrifuged, washed with Amphibian Ringer's solution (Coldspring Harbor), centrifuged and suspended in one ml of 0.027M Na_3Citrate for 10 minutes. Cells were centrifuged at 125 g for five minutes and the pellet was resuspended in methanol: acetic acid (3:1), centrifuged, and washed a further two times in methanol: acetic acid (3:1) prior to storage overnight at 4 °C. The next day, conventional drop-splash technique was performed and cells were cover-slipped with Gelvatol mounting medium (Cold Spring Harbor Protocols) containing 1 µg/ml DAPI. Homologous chromosomes were paired and arranged according to size, with the longest pair being designated as Chromosome 1 = 2n. Image J software with the Levan plugin was used to measure chromosome arm length and long arm to short arm ratios of 1–1.69, 1.7–2.99 and 3–6.99, respectively, were used to designate metacentric, submetacentric and subtelocentric configurations (Levan et al. 1964; Sakamoto and Zacaro 2009).

An Olympus BX60 microscope, colour CCD Leica DFC425C camera, and EL-6000 Leica light source were used for imaging at 1000 x magnification under oil immersion. Leica LAS-AF software was used to capture images.

Table 1. Measurements of *L. spenceri* chromosomal arm ratios and corresponding chromosomal morphology designations. Chromosomes where both homologous chromosomes display a DAPI negative region are indicated with an *. Chromosomes where only one matched chromosome of that pair displays a DAPI negative region are indicated with a #. Metacentric, submetacentric, and subtelocentric chromosomal designation are defined as a long arm to short arm ratios of 1–1.69, 1.7–2.99, and 3–6.99, respectively.

	Chromosome Number						
	1	3	4	4	5	6	7
Southern Site Female	1.69 ± 0.1 metacentric	2.24 ± 0.3 submetacentric	3.27 ± 0.6 subtelocentric	1.52 ± 0.2 metacentric	3.76 ± 0.54 subtelocentric	2.40 ± 0.8 submetacentric	2.16 ± 0.3 submetacentric
Southern Site Male	1.50 ± 0.2 metacentric	2.39 ± 0.5 submetacentric	3.61 ± 0.9 subtelocentric	1.58 ± 0.3 metacentric	4.05 ± 1.1 subtelocentric	2.05 ± 0.3 submetacentric	1.89 ± 0.3 submetacentric
Northern Site Adult	1.62 ± 0.1 metacentric	2.11 ± 0.2 submetacentric	3.79 ± 0.5 subtelocentric	1.63 ± 0.2 metacentric	3.60 ± 0.3 subtelocentric	2.10 ± 0.3 submetacentric	2.01 ± 0.3 submetacentric
Central Site, juvenile 1	1.60 ± 0.1 metacentric	1.98 ± 0.4 submetacentric	3.17 ± 0.9 subtelocentric	1.51 ± 0.1 metacentric	3.05 ± 0.4 subtelocentric	2.13 ± 0.5 submetacentric	2.09 ± 0.4 submetacentric
Central Site, juvenile 2	1.62 ± 0.2 metacentric	1.87 ± 0.3 submetacentric	3.17 ± 0.5 subtelocentric	1.35 ± 0.2 metacentric	3.57 ± 0.4 subtelocentric	2.02 ± 0.3 submetacentric	2.00 ± 0.1 submetacentric
Northern Site x Central Site cross	1.64 ± 0.2 metacentric	1.79 ± 0.1 submetacentric	3.26 ± 0.5 subtelocentric	1.42 ± 0.2 metacentric	3.44 ± 0.4 subtelocentric	2.41 ± 0.3 submetacentric	1.81 ± 0.4 submetacentric

	Chromosome Number					
	8	9	10	11	12	13
Southern Site Female	2.53 ± 0.7 submetacentric	1.73 ± 0.5 submetacentric *	1.5 ± 0.1 metacentric	1.49 ± 0.3 metacentric	1.28 ± 0.6 metacentric	1.44 ± 0.2 metacentric
Southern Site Male	2.92 ± 0.6 submetacentric	2.08 ± 0.3 submetacentric *	1.30 ± 0.2 metacentric	1.41 ± 0.2 metacentric	1.50 ± 0.4 metacentric	1.18 ± 0.1 metacentric
Northern Site Adult	2.65 ± 0.7 submetacentric	2.22 ± 0.3 submetacentric *	1.23 ± 0.1 metacentric	1.24 ± 0.1 metacentric	1.20 ± 0.1 metacentric	1.57 ± 0.4 metacentric
Central Site, juvenile 1	2.43 ± 0.6 submetacentric	2.11 ± 0.4 submetacentric *	1.65 ± 0.5 metacentric	2.56 ± 0.5 submetacentric *	1.60 ± 0.3 metacentric	1.57 ± 0.4 metacentric
Central Site, juvenile 2	2.03 ± 0.2 submetacentric	2.01 ± 0.2 submetacentric *	1.44 ± 0.3 metacentric	2.12 ± 0.4 submetacentric *	1.66 ± 0.4 metacentric	1.59 ± 0.2 metacentric
Northern Site x Central Site cross	1.98 ± 0.5 submetacentric	1.81 ± 0.4 submetacentric *	1.34 ± 0.2 metacentric	2.35 ± 0.5 submetacentric #	1.41 ± 0.2 metacentric	1.34 ± 0.3 metacentric

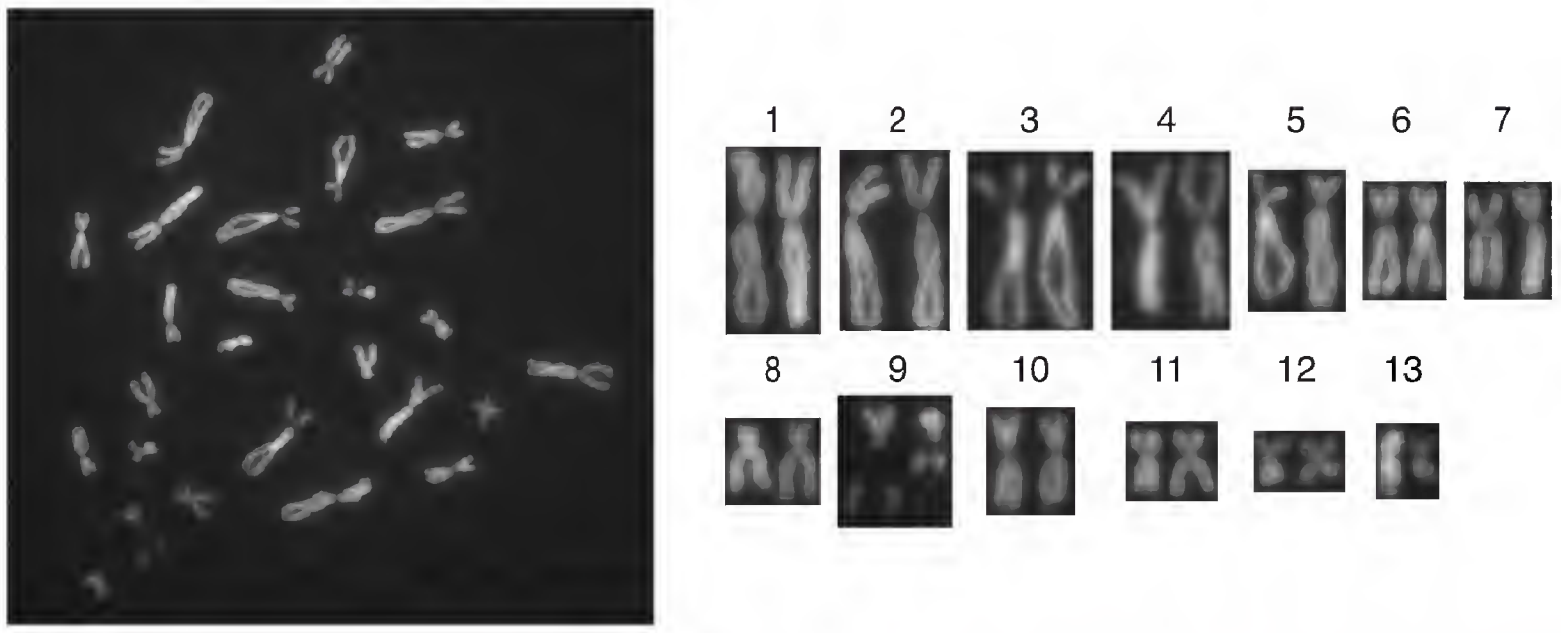

Fig. 2. Karyotype from a Southern Site *L. spenceri* adult female. A representative karyotype demonstrates the *L. spenceri* 2n = 26 karyotype and a DAPI negative area in the long arm of chromosome 9.

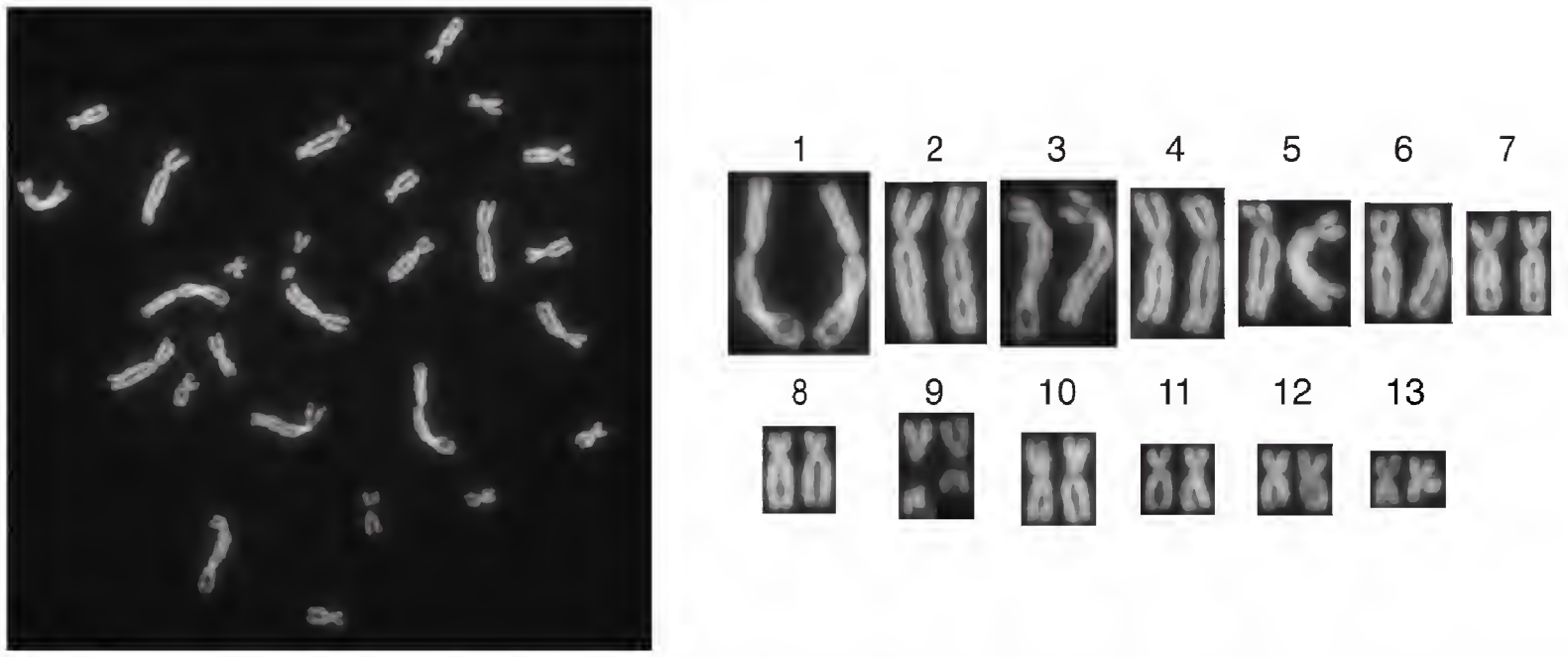

Fig. 3. Karyotype from a Southern Site *L. spenceri* adult male. A representative karyotype demonstrates the *L. spenceri* 2n = 26 karyotype and a DAPI negative area in the long arm of chromosome 9.

Results

Litoria spenceri, Southern Site: field study adult specimens

One female and one male frog were studied from the Southern Site. Minimal numbers of animals were used in this study due to the critically endangered nature of this species, the lack of readily available tissue samples, and the unwillingness to harm such critically endangered animals explicitly for use in experimentation. Twenty of 23 spreads counted were 2n = 26 for the female and nine of 11 spreads in the male. Eight prepared karyotypes (chromosomes lined up in order) from the female and seven prepared karyotypes from the male confirmed the 2n = 26 karyotype, and arranged in descending order of size, revealed four larger, three medium, and six smaller chromosome pairs (Figs. 2, 3, and data not shown). DAPI negative regions were evident on the long arms of chromosome 9 for each sex in all karyotypes (Figs. 2, 3, 4, and data not shown). Chromosomes 1, 4, 10, 11, 12, and 13 appear metacentric, chromosomes 2, 6, 7, 8, and 9 appear submetacentric, and chromosomes 3 and 5 appear subtelocentric (Table 1).

Litoria spenceri, Northern Site: captive bred adult specimen

One unsexed frog was studied from the Northern Site. Eight of 12 spreads counted were 2n = 26. The same chromosomal metacentric, metacentric, and subtelocentric configuration was observed as for the Southern Site animal karyotypes, with DAPI negative regions again evident on the long arms of chromosome 9 in four karyotypes that were prepared (Figs. 4, 5; Table 1 and data not shown).

Litoria spenceri, Central Site: captive bred juvenile specimens

Two unsexed juvenile frogs were studied from the Central Site. For one frog, 30 of 35 spreads counted were 2n = 26 and 17 of 24 in the other. For one frog, five karyotypes were prepared and for the other, four karyotypes were prepared (Figs. 6 and 7; data not shown). Chromosomal configurations differed slightly compared to those from animals originating from both the Southern and Northern sites, with DAPI negative regions being evident on the long arms of chromosomes 9 and 11 for both animals in all karyotypes (Figs. 4, 6, 7, and

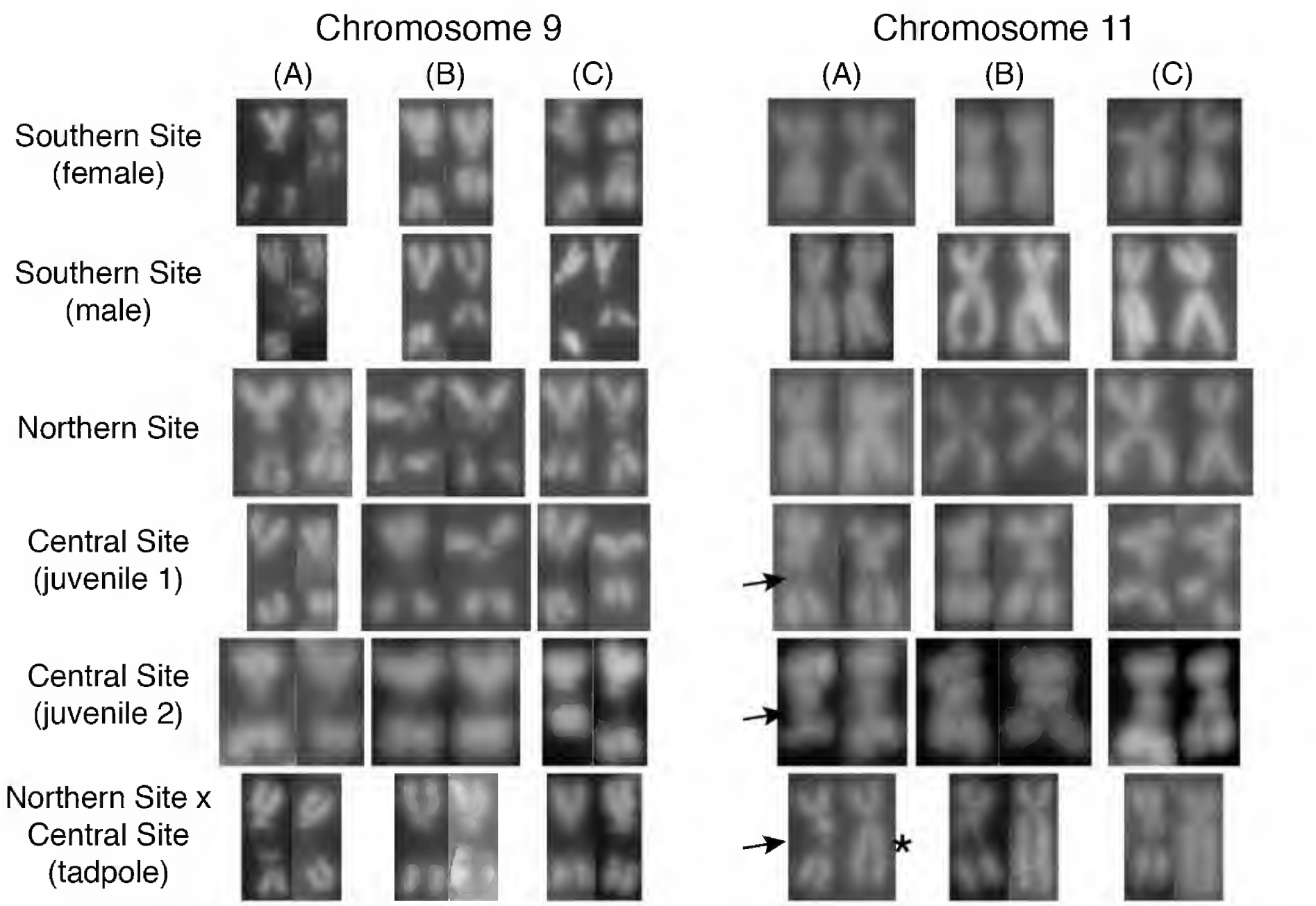

Fig. 4. Chromosomes 9 and 11 from different *L. spenceri* populations. Three representative chromosomes from each animal demonstrate a highly conserved DAPI negative region in the long arms of chromosome 9. A DAPI negative region is observed in the long arm of chromosome 11, but only in the Central Site juveniles and in only one matched chromosome of the Northern Site x Central Site tadpole hybrid. Arrows indicate the chromosome 11 DAPI negative region. Asterisks indicate the paired submetacentric chromosome 11 matched pair of the Northern Site x Central Site tadpole hybrid.

data not shown; Table 1). Chromosome 11 here also differed and was submetacentric and not metacentric.

***Litoria spenceri* Northern Site x Central Site: captive bred tadpole**

One unsexed mixed Northern and Central Site tadpole was studied. Thirty-one of 33 spreads counted were 2n = 26. Nine karyotypes were prepared, confirming the 2n = 26 karyotype (Fig. 8; data not shown). When arranged in descending order of size, the same chromosomal metacentric, submetacentric, and subtelocentric configuration was observed as for the Central Site animals (Figs. 4, 8; Table 1; data not shown). DAPI negative regions were evident on the short arms of both homologous chromosomes of chromosome 9, and one chromosome 11 matched chromosome in all nine karyotypes (Figs. 4, 8; data not shown). The one matched chromosome of the chromosome 11 pair that did not display this DAPI negative region was submetacentric in all nine karyotypes (Figs. 4, 8; data not shown).

Discussion

These data demonstrate a 2n = 26 karyotype for *L. spenceri*. This karyotype is characteristic of all species of the genus *Litoria* described to date, with the exception of *L. infrafrenata* which has a 2n = 24 karyotype (see King 1980; Mollard 2018a). The *L. spenceri* karyotypes described here, display the highly conserved *Litoria* genus centromere positions and corresponding arms ratios, with the characteristic: pairs 1 and 4 metacentric, pairs 2 and 6 submetacentric and pairs 3 and 5 acrocentric chromosomal morphologies (see King 1980). These data further demonstrate a consistent DAPI negative region approximately midway on the long arms of chromosome 9 of all animals studied. A DAPI negative region approximately midway on chromosome 11 was also observed only in representatives of the Central Site population and apparently inherited in a heterozygous state in a Northern Site x Central Site hybrid cross tadpole.

The described DAPI negative regions are likely NORs, where undercondensation or despiralization of rDNA is known to typically result in this type of relative DAPI understaining (see Haaf et al. 1984; McStay 2016). NORs are regions of chromosomes that contain ribosomal DNA genes, usually present as tandem repeats, that code for the rRNA of interphase nucleoli. Amphibian NORs have been traditionally detected using a specific silver staining method and proximal chromo-

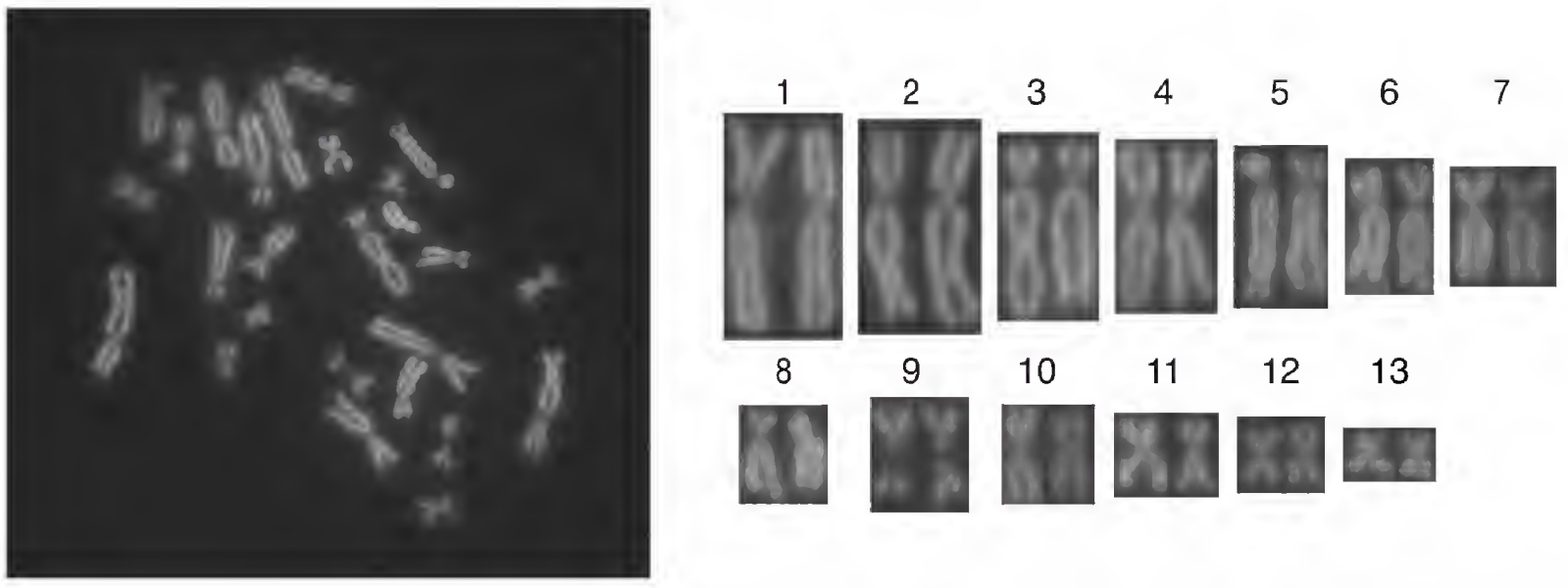

Fig. 5. Karyotype from a Northern Site *L. spenceri* unsexed adult. A representative karyotype demonstrates the *L. spenceri* 2n = 26 karyotype and a DAPI negative area in the long arm of chromosome 9.

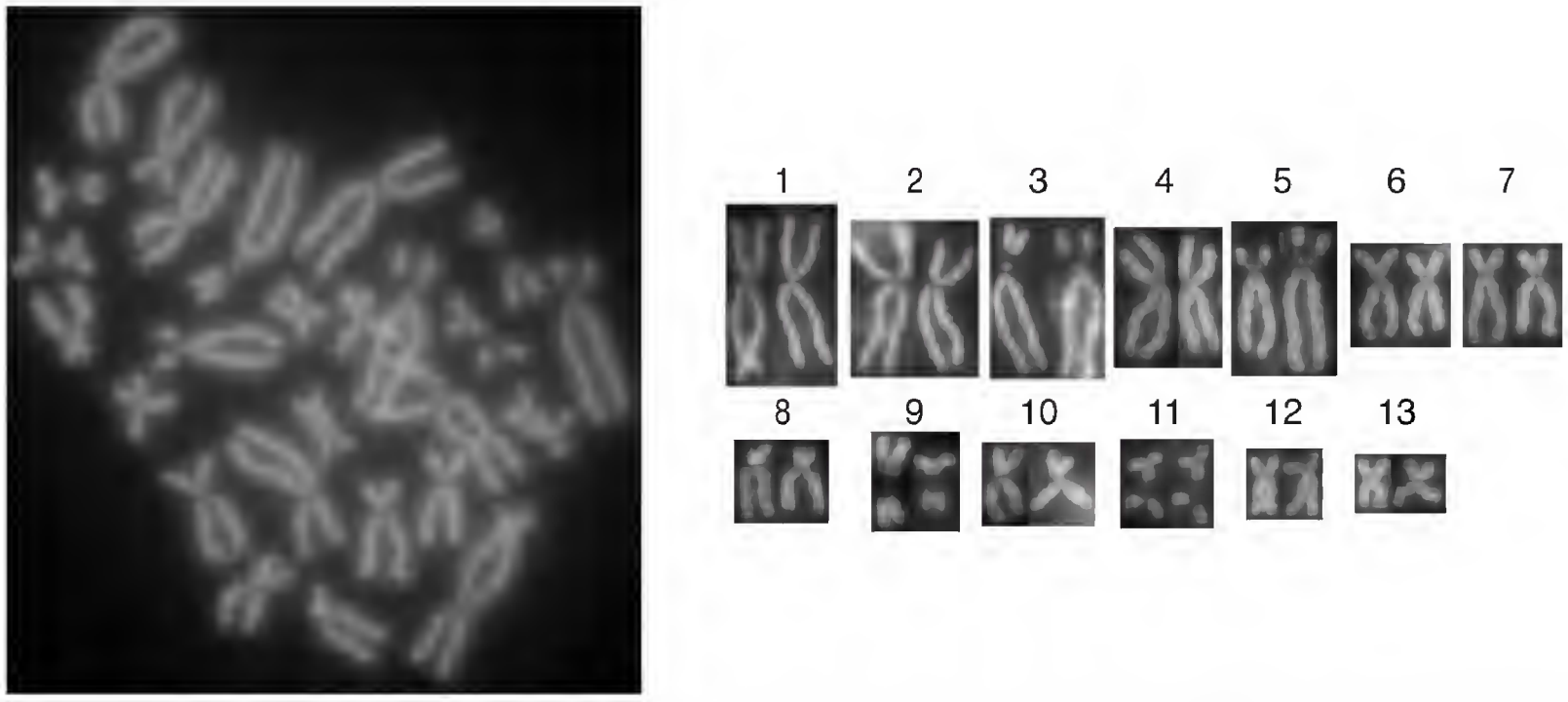

Fig. 6. Karyotype from a Central Site *L. spenceri* unsexed juvenile, animal 1. A representative karyotype demonstrates the *L. spenceri* 2n = 26 karyotype and DAPI negative areas in the long arms of chromosomes 9 and 11.

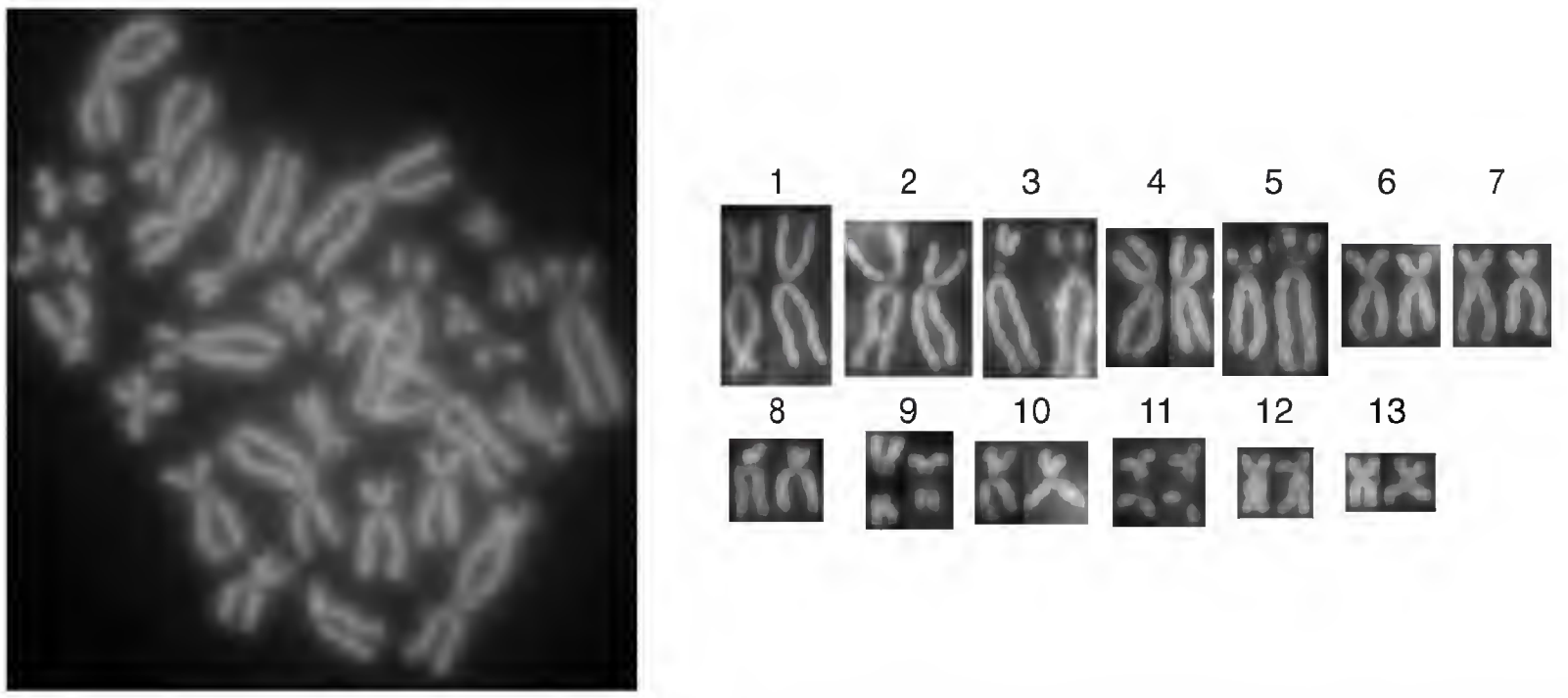

Fig. 7. Karyotype from a Central Site *L. spenceri* unsexed juvenile, animal 2. A representative karyotype demonstrates the *L. spenceri* 2n = 26 karyotype and DAPI negative areas in the long arms of chromosomes 9 and 11.

somal arms visualized with orcein or Giemsa counter-stains (see Bloom and Goodpasteur 1976; Mahony and Robinson 1986). Visually apparent DAPI-induced under-condensations in chromosomal regions strongly overlap with silver stained NORs detected using these methods, meaning that DAPI staining represents a good proxy for their identification (Haaf et al. 1984; McStay 2016). In studies that have investigated the location of the NOR in anurans, and specifically the Australian tree frogs of the genus *Litoria*, the NOR location is extremely well conserved and almost always located in the same region of the same chromosome pair in closely related species complexes (King 1980; Schmid 1983; King et al. 1990). Phylogenetic studies have placed *L. spenceri* within a

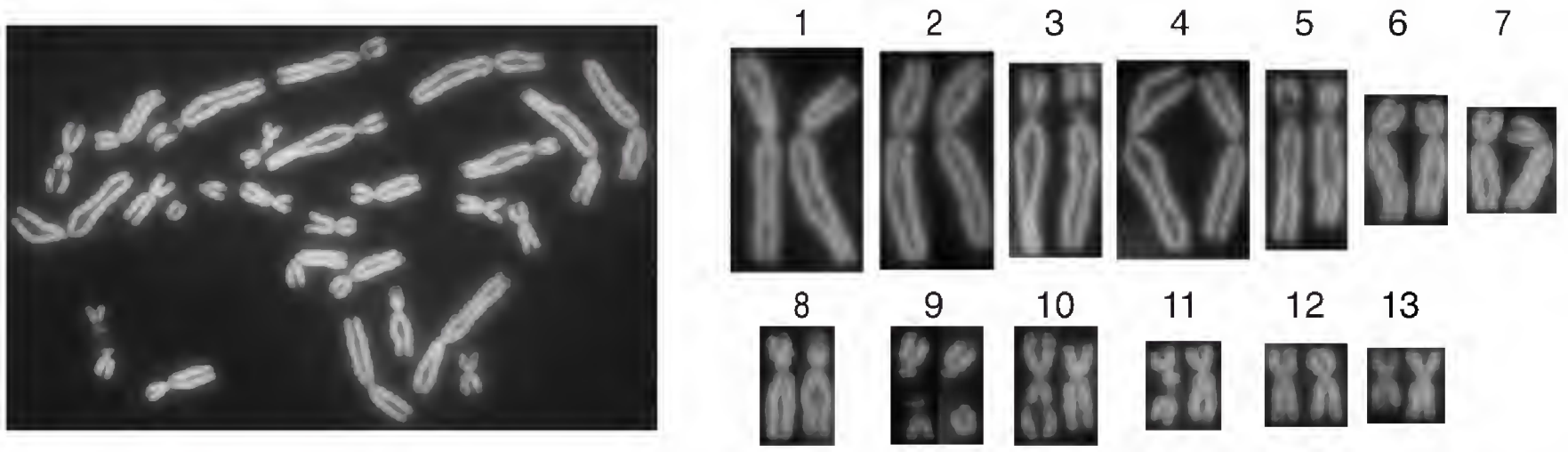

Fig. 8. Karyotype from a Northern Site x Central Site *L. spenceri* unsexed tadpole hybrid. A representative karyotype demonstrates the *L. spenceri* 2n = 26 karyotype and DAPI negative areas in the long arm of chromosome 9, as well as one matched chromosome of chromosome 11. The chromosome 11 matched chromosome that does not contain the DAPI negative area is submetacentric.

closely related species group with *L. pearsoniana* (*L. pearsoni*) and *L. phyllochroa* and both these latter two animals display an NOR on the long arms of chromosome 9 (Donnellan et al. 1999; McGuigan et al. 1998). The notion that the DAPI negative region of *L. spenceri* chromosome 9 described here is an NOR, therefore, is strongly supported.

Although species of the genus *Limnodynastes* may contain up to four NORs, most *Litoria* species contain one, with exceptions being *L. phyllocroa*, allocated to a closely related species group with *L. spenceri*, and *L. raniformis*, which both contain two (King 1980, 1990; Mahony and Robinson 1986). With the previous demonstration of a second NOR in *L. phyllochroa*, it is suggested that the chromosome 11 DAPI negative region is an NOR representing a distinguishing marker of the Central Site *L. spenceri* subpopulation. This suggestion is supported by the findings that: (i) NORs are hereditable in the heterozygous state, and (ii) the one hybrid tadpole chromosome 11 homologous chromosomal pair contained such a DAPI negative region (see King 1980; Stults et al. 2008). The presence of a life-stage specific chromosomal restriction is unprecedented. Further, this chromosome 11 DAPI negative region was not present in either the female or male Southern Site wild or Northern Site captive specimens. Together, these observations suggest it is neither life stage-, sex- nor captivity-linked.

Therefore, representatives of all three species of this closely related group for which karyotypes have been presented can apparently display different NOR configurations: *L. pearsoniana* and *L. spenceri*, apparently mid-way on the long arm of chromosome 9; *L. phyllochroa* apparently distal on the long arm of chromosome 9; *L. pearsoniana* with an additional NOR apparently distal on the short arm of chromosome 8, and the Central Site *L. spenceri* subpopulation, apparently mid-way on the long arm of chromosome 11 (Mahony and Robinson 1986; Schmid et al. 2002).

Conclusion

Further studies to unequivocally identify: (i) these DAPI negative regions as NORs and (ii) an apparent metacentric to submetacentric chromosome 11 conversion in the Northern Site x Central Site tadpole hybrid are warranted (see Mahony and Robinson 1986; Zalesna et al. 2017). Regardless, these DAPI negative regions represent distinguishing markers of *L. spenceri* subpopulations that may assist with animal husbandry techniques. Using the techniques described here can help identify subpopulations of endangered amphibian species. Therefore, adding karyotyping with DAPI staining for NORs as part of biobanking conservation management programs is an important step in ensuring that specific populations and their unique traits are preserved.

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